

Essential Role for Estrogen in Protection against *Vibrio vulnificus*-Induced Endotoxic Shock

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Received 30 January 2001/Returned for modification 8 May 2001/Accepted 11 July 2001

Little is known about the underlying mechanisms that result in a sexually dimorphic response to *Vibrio vulnificus* endotoxic shock. *V. vulnificus* is a gram-negative bacterium, considered one of the most invasive and rapidly fatal human pathogens known. However, 85% of individuals that develop endotoxic shock from *V. vulnificus* are males. Using the rat, we have developed a model for *V. vulnificus* endotoxic shock that mimics the sexually dimorphic response in humans. Gonadectomy in females results in increased mortality, and estrogen replacement results in decreased mortality in both gonadectomized males and females. These results demonstrate that estrogen is providing protection against *V. vulnificus* lipopolysaccharide-induced endotoxic shock.

The Centers for Disease Control and Prevention have estimated that foodborne diseases cause approximately 5,000 deaths in the United States each year (16). Seventy-two percent of all deaths from foodborne transmission are bacterial in origin. Endotoxic shock occurs following infection by gram-negative bacteria and results in nearly half of the deaths of patients with sepsis. One way individuals become infected is through ingestion of contaminated food. *Vibrio vulnificus* is a gram-negative bacterium that can produce endotoxic shock following the consumption of raw shellfish and is considered one of the most invasive and rapidly fatal human pathogens known. Fatality rates of over 60% with times to onset of symptoms ranging from as little as 7 h to several days have been reported (23). Unique aspects of the infections caused by this bacterium are that most cases occur in persons over the age of 50 (23), and, based on data compiled by the Food and Drug Administration, of 249 cases that occurred in this country over the last 10 years, 85% of the individuals who developed endotoxic shock from *V. vulnificus* infection were males (M. Bashin, personal communication).

Although both endotoxic shock and the mechanisms of pathogenesis of *V. vulnificus* are areas of intense investigation, the role of gender in *V. vulnificus*-induced endotoxic shock and death is not understood. This sexually dimorphic response to *V. vulnificus* endotoxic shock may be due to the differences in hormonal profiles between males and females. It is well established that estrogen produces a variety of effects in females, ranging from mediating ovulation and implantation to regulation of immune function and the cardiovascular system (10, 19, 22, 26, 30, 31).

The effects of estrogen in regulating the responsiveness of females to lipopolysaccharide (LPS)-induced endotoxic shock have not been well studied. In 1965, Nolan and O'Connell (22) described experiments in which female blood altered normal vasoconstrictive responses to endotoxin in isolated rat livers

exposed to bacterial LPS. Nolan then followed this with a report in 1967 (21) demonstrating that pretreatment with pharmacological doses of conjugated estrogens for up to 1 h prior to LPS exposure protected rats from the lethal effects of endotoxin. A more recent study showed exogenous estradiol decreased the percentage of endotoxin-induced deaths in male rats in a dose-dependent manner (7). Another study has demonstrated that exogenous estradiol given 1 h after LPS restored cardiac output in male rats (24). Thus, estrogen appears to be protective when given prior to or immediately after exposure to endotoxin. However, previous work does not demonstrate that females are normally protected from the lethal effects of endotoxin by circulating levels of estrogens. We hypothesized that the decreased mortality from *V. vulnificus* endotoxic shock in women is due to their higher estrogen levels.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats, bred in-house from Charles River Co. (Fayetteville, N.C.) stock, were used. All animals were at least 8 weeks of age and weighed at least 200 g when injected with LPS. Male and female rats were placed into one of three treatment groups: LPS injection only; gonadectomy and LPS injection; and gonadectomy, LPS injection, and estrogen treatment. Androgenized and mock-androgenized females were produced by injecting testosterone propionate subcutaneously (s.c.; 200 ng/g of body weight in corn oil) or corn oil s.c. on postnatal day 5.

Chemicals and hormones. All chemicals and hormones were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted.

LPS. The LPS portion of the bacterium *Vibrio vulnificus* (strain c7184K, opaque variant) was prepared according to the method of Hitchcock and Brown (11), with the modifications recommended by Preston and Penner (28). This method has been widely used and accepted for analysis of LPS from a variety of bacteria (1, 2, 13, 18, 25), especially that from marine bacteria (4, 27, 32). Briefly, the cells were grown overnight at 37°C on heart infusion agar. Cells were scraped, weighed, placed in phosphate-buffered saline, and stored at 4°C for up to 7 days. Cells were placed in buffer solution (125 ml/20 g of cells) consisting of 80% Tris solution (62.5 mM), 10% glycerol, 0.5% sodium dodecyl sulfate, and 0.5% β -mercaptoethanol. The solution was heated to 100°C for 15 min and then cooled to 60°C. Crude proteinase K (59.6 mg) was added, and the solution was incubated at 55°C overnight and then heated to 100°C for 15 min to denature the proteinase K. The solution was dialyzed against distilled H₂O at 2°C until no odor of β -mercaptoethanol could be detected. The solution was then lyophilized and stored at –80°C.

Surgeries. Gonadectomies were performed when animals reached 8 weeks of age and 200 g of body weight. Rats were anesthetized with 2.5% Avertin (0.017 ml/g of body weight) to a level sufficient to inhibit the toe jerk reflex. Ovariec-

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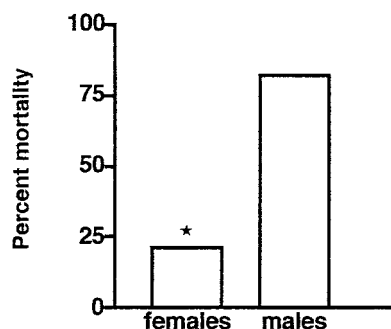


FIG. 1. Mortality rate in female and male rats. Female ($n = 19$) and male ($n = 11$) rats were injected intravenously with *V. vulnificus* LPS (30 to 50 $\mu\text{g/g}$ of body weight). Mortality was determined as those animals that died within 24 h. A star indicates that statistically significant differences were found when chi-square analysis of the data was performed followed by the sequential Bonferroni procedure ($P < 0.05$).

tomies were performed through dorsolateral incision. Orchidectomies were performed through bilateral scrotal incision. A representative sample of rats was bled through orbital bleeds 1 month after gonadectomies. A radioimmunoassay of the serum (Binax, Portland, Maine) was then performed to ensure that all endogenous gonadal hormones had been eliminated.

Injections. For each batch of LPS, a dose-response curve was determined, and subsequent experiments were done with a concentration of LPS that resulted in 80% mortality in males. The *V. vulnificus* LPS (30 to 50 mg/kg of body weight) was administered to rats under general anesthesia (2.5% avertin) through tail vein injection. Females were injected only when in estrus, as determined by microscopic observation of vaginal smears. Following administration of LPS, rats were observed over a period of 24 h, and mortality was recorded. Estradiol-17 β (s.c., 0.5, 1.0, or 2.0 μg in corn oil) was administered for 5 days, followed by LPS injection on day 5.

Statistics. Comparison of percentage survival data across groups were conducted via contingency table analyses, in which the sequential Bonferroni procedure was used to interpret probabilities associated with the chi-square statistics from the contingency tables (29).

RESULTS

Influence of gender on mortality in response to *V. vulnificus* LPS. As shown in Fig. 1, within a 24-h period, female rats injected with LPS died at a significantly lower rate than males (21% versus 82%; $P < 0.05$). This suggests that sexually mature males and sexually mature females in the estrus phase of their hormonal cycle have different susceptibilities to *V. vulnificus* LPS. Females were injected in the estrus phase following exposure to the preovulatory estrogen surge, suggesting that this alteration in susceptibility might be attributed to hormonal differences.

Influence of estrogen on mortality in females. As shown in Fig. 2, long-term ovariectomized females died at a significantly higher rate than normal females (75% versus 21%; $P < 0.05$) and at a rate similar to that of normal males (75% versus 80%). Sera from ovariectomized females had no detectable estrogen (data not shown). To determine if exogenous estrogen administration could protect ovariectomized females from death due to LPS, ovariectomized animals were treated with estradiol-17 β for 5 days and injected with LPS on day 5. Following 5 days of estrogen exposure (2 $\mu\text{g/day}$), mortality rates of ovariectomized females were no longer different from those of normal females (38% versus 21%; $P = 1$). This protective effect of estrogen was shown to be dose dependent (Fig. 2). In addition,

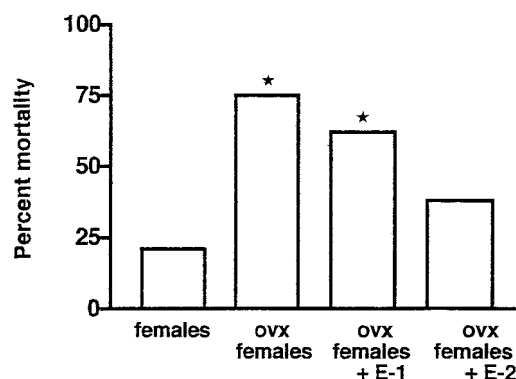


FIG. 2. Mortality rate in females with and without estrogen exposure. Female rats ($n = 19$), ovariectomized females (ovx females; $n = 20$), ovariectomized females injected with 1 μg of estradiol-17 β per day for 5 days (ovx females E-1; $n = 21$), and ovariectomized females injected with 2 μg of estradiol-17 β per day for 5 days (ovx females E-2; $n = 16$) were injected intravenously with *V. vulnificus* LPS (30 to 50 $\mu\text{g/g}$ of body weight). Mortality was determined as those animals that died within 24 h. Stars indicate that statistically significant differences were found between mortality in the ovariectomized and ovariectomized females injected with 1 μg of estradiol-17 β per day when compared to untreated females, but not between ovariectomized females injected with 2 μg of estradiol-17 β per day and untreated females when chi-square analysis of the data was performed followed by the sequential Bonferroni procedure ($P < 0.05$).

androgenized females were used as another model lacking estrogen, because they never cycle, are in persistent estrus, and thus have never been exposed to endogenous gonadal estrogen or progesterone (6). When injected with LPS they died at a rate significantly higher than that of mock-androgenized females in estrus (90% versus 46%, $P < 0.01$; Fig. 3). These data suggest that estrogen in females provides protection from death due to *V. vulnificus*-induced endotoxemia.

Influence of estrogen on mortality in males. As shown in Fig. 4, orchidectomized males died at a rate similar to that of normal males. Orchidectomized males had no detectable serum testosterone (data not shown). To determine if estrogen

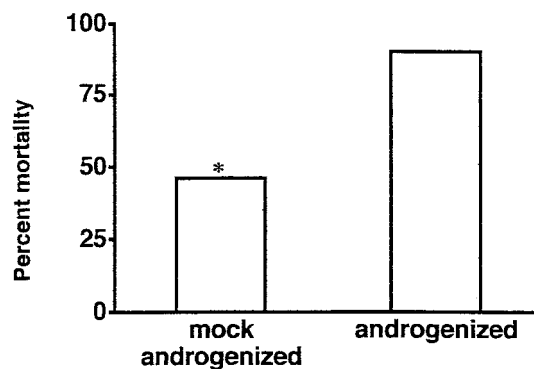


FIG. 3. Mortality rate in mock-androgenized and androgenized females. Mock-androgenized ($n = 16$) and androgenized ($n = 20$) rats were injected intravenously with *V. vulnificus* LPS (30 to 50 $\mu\text{g/g}$ of body weight). Mortality was determined as those animals that died within 24 h. An asterisk indicates that statistically significant differences were found when chi-square analysis of the data was performed followed by the sequential Bonferroni procedure ($P < 0.05$).

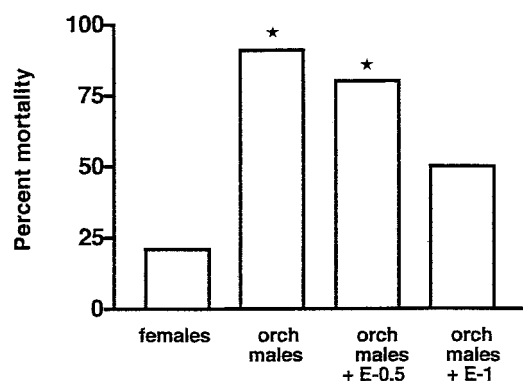


FIG. 4. Mortality rate in males with and without estrogen exposure. Female rats ($n = 19$), orchidectomized male rats (orch male; $n = 11$), orchidectomized males injected with $0.5 \mu\text{g}$ of estradiol- 17β per day for 5 days (orch male E-0.5; $n = 10$), and orchidectomized males injected with $1 \mu\text{g}$ of estradiol- 17β per day for 5 days (orch male E-1; $n = 10$) were injected intravenously with *V. vulnificus* LPS (30 to $50 \mu\text{g/g}$ of body weight). Mortality was determined as those animals that died within 24 h. Stars indicate that statistically significant differences were found between mortality in the orchidectomized males and that in orchidectomized males injected with $0.5 \mu\text{g}$ of estradiol- 17β per day when compared to untreated females, but not between orchidectomized males injected with $1 \mu\text{g}$ of estradiol- 17β per day and untreated females when chi-square analysis of the data was performed followed by the sequential Bonferroni procedure ($P < 0.05$).

could protect males from death due to endotoxemia, orchidectomized males were treated with estrogen for 5 days and injected with LPS on day 5. As the dose of estrogen increased from $0.5 \mu\text{g/day}$ to $1 \mu\text{g/day}$, the mortality rate of the orchidectomized males decreased (from 80% to 50%), indicating that the protective effect of estrogen is dose dependent (Fig. 3). Orchidectomized males treated with $1 \mu\text{g}$ of estrogen died at a rate not different from that of normal females (50% versus 21%). This decreased mortality rate was not quite significantly different from that of normal males (82% versus 50%). These data provide evidence that estrogen protects females and can protect males from mortality in response to *V. vulnificus*-induced endotoxic shock.

DISCUSSION

Gender-based differences have been noted in the outcome of sepsis resulting from ingestion of *V. vulnificus* (23). Although such gender differences have been reported clinically, there are currently no animal models that have been designed to investigate this phenomenon. The *V. vulnificus* endotoxin model presented here employed LPS prepared by an extraction method that did not involve the phenol extraction step frequently employed in other methods, and thus our results could reflect to some extent the presence of non-LPS contaminants present in our extract. However, our model almost exactly mimics the mortality rates observed following *V. vulnificus* infections in humans, and thus allowed us to investigate this sexually dimorphic response.

It is well established that estrogen produces a variety of effects in females, ranging from mediating ovulation and implantation to regulation of immune function and the cardiovascular (reviewed in references 21, 22, and 31). Although

estrogen can influence a variety of responses, its influence on regulating the responsiveness of females to *V. vulnificus* LPS-induced endotoxic shock has never been studied. The increased mortality in females that have been ovariectomized or androgenized and have no circulating estrogen suggests that the increased susceptibility is due to either the loss of ovarian estrogens, some other ovarian product, or a combination of both. It is evident that the major factor in decreasing susceptibility is circulating estrogens, because exogenous administration of a physiological dose of estrogen for 5 days to long-term ovariectomized females restores resistance to endotoxic shock, as demonstrated by the significant decrease in mortality. It is possible that ovarian progesterone may enhance the effects of estrogen and that a combination of estrogen and progesterone would be even more effective in providing protection against *V. vulnificus* endotoxic shock.

Experiments with orchidectomized males given exogenous estrogen further establish the role of estrogen as a mediator of resistance to endotoxic shock. These males have no detectable circulating levels of testosterone, and when given estrogen exogenously, their mortality rates decrease and are no longer different from those of normal females or estrogen-treated ovariectomized females. The decreased mortality is dose dependent, and mortality is expected to be reduced significantly from that of normal males as the amount of estrogen increases. It is interesting to note that orchidectomized males appear to die at a higher rate than normal males, although mortality rates were not significantly different. We believe that if lower concentrations of LPS were given to orchidectomized males, their mortality rates would have been significantly higher than those of normal males. This would not be surprising given that testosterone can be aromatized to estrogen and thus is a source of estrogen for males. Following orchidectomy, males would have decreased testosterone as well as decreased estrogen levels and thus would have increased mortality rates. Future studies must be done to verify that mortality increases with orchidectomy and that this is due to loss of estrogen and not testosterone. It is certainly possible that both sex steroids provide protection, but the responses to estrogen are greater and thus result in the sexually dimorphic response to *V. vulnificus* LPS.

It is well documented that both males and females with liver disease have disturbances in steroid metabolism. The main alteration in sex hormone metabolism consists of elevated estrone and sex hormone binding globulin concentrations in both males and females (5, 12). Although estrogen levels are elevated, this reflects an increase in estrone, a less active form of estrogen than the main ovarian estrogen, estradiol. In addition, it has been demonstrated that patients with chronic liver diseases have significantly lower hepatic estrogen receptor concentrations, and this reduction is determined by the degree of liver dysfunction. Thus, although liver disease increases overall estrogen levels in males and females, it is primarily due to increases in estrone, and not the more active estradiol. This, in conjunction with decreased hepatic estrogen receptor levels, could explain why males with liver disease are not protected as a result of altered hormone metabolism.

It must be considered whether the disproportionate number of males who die from *V. vulnificus* can be accounted for simply because more men than women eat raw oysters, and more men than women have liver disease (the major risk factor for infec-

tion). In a study reported by Desenclos et al. (8), males were twice as likely as females to report being raw oyster eaters (44% versus 22% [odds ratio, 2.7; 95% confidence interval, 2.1 to 3.5]). Furthermore, according to a recently reported study conducted by the National Institutes of Health (1), of persons reporting alcohol dependence, 1.96% of males and 1.27% of females reported enlarged liver, yellow jaundice, cirrhosis, hepatitis, or other liver disease. Thus, the ratio of males to females reporting liver disease is estimated at ca. 1.5:1. Taken together, the oyster consumption and liver disease data indicate that men are ca. three times more likely to develop infection with *V. vulnificus* than are women. While this is significant, it is not sufficient to explain the epidemiologic data indicating a male/female case ratio of ca. 6:1.

The mechanism by which estrogen provides protection against *V. vulnificus* LPS-induced endotoxic shock is unknown. However, others have clearly demonstrated that estrogen has effects on both the function of immune cells as well as the cardiovascular system. Our previous studies have shown that nitric oxide (NO) is a mediator of endotoxic shock (9, 15) produced by *V. vulnificus* and that inhibition of NO production enhances cardiovascular function and thus decreases mortality (3). In addition, specific cytokines like tumor necrosis factor α are known to be important regulators of immune function and can also interact to effect cardiovascular responses (3, 14, 20). Both of these soluble regulators of cell function have been shown to be regulated by estrogen and thus may play a role in altering susceptibility to *V. vulnificus* LPS-induced endotoxic shock (17, 33) in males and females. Further investigation will be required to reveal the exact nature of the response to testosterone and progesterone and to determine the mechanisms by which estrogen provides protection.

ACKNOWLEDGMENTS

We thank Larry Leamy for valuable contributions to the manuscript. This work was supported by NIH grant R15-AI/OD37822-01A1.

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